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# Early life adversity affecting the attachment bond alters ventral tegmental area transcriptomic patterning and behavior almost exclusively in female mice

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ABSTRACT

Early life experiences that affect the attachment bond formation can alter developmental trajectories and result in pathological outcomes in a sex-related manner. However, the molecular basis of sex differences is quite unknown. The dopaminergic system originating from the ventral tegmental area has been proposed to be a key mediator of this process.

Here we exploited a murine model of early adversity (Repeated Cross Fostering, RCF) to test how interfering with the attachment bond formation affects the VTA-related functions in a sex-specific manner.

Through a comprehensive behavioral screening, within the NiH RDoC framework, and by next-generation RNA-Seq experiments, we analyzed the long-lasting effect of RCF on behavioral and transcriptional profiles related to the VTA, across two different inbred strains of mouse in both sexes.

We found that RCF impacted to an extremely greater extent VTA-related behaviors in females than in males and this result mirrored the transcriptional alterations in the VTA that were almost exclusively observed in females. The sexual dimorphism was conserved across two different inbred strains in spite of their divergent long lasting consequences of RCF exposure.

Our data suggest that to be female primes a sub-set of genes to respond to early environmental perturbations.

This is, to the best of our knowledge, the first evidence of an almost exclusive effect of early life experiences on females, thus mirroring the extremely stronger impact of precocious aversive events reported in clinical studies in women.

## 1. Introduction

The early postnatal period represents a critical time window for individuals' neurodevelopment, during which programmed trajectories are highly sensitive to environmental influences (Daskalakis et al., 2013; Luby et al., 2020; Nelson and Gabard-Durnam, 2020; Babicola et al., 2021). In particular, the mother-pup attachment bond plays a critical role in offspring's future and pup's brain development (Bowlby, 1982). Early experiences affecting the attachment bond formation can result in maladaptive behaviors or, more extremely, mental illness (Heim et al.,

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2010; Opendak and Sullivan, 2016; 2017, 2020; Bryant, 2016; Maccari et al., 2014, 2017; Di Segni et al., 2018; Song and Gleeson, 2018; Torres-Berrío et al., 2019).

One mechanism by which early experiences can lead to mental disorders vulnerability is by affecting dopaminergic neurocircuitry (D'Addario et al., 2021; Hollon et al., 2015; Ironside et al., 2018; Russo and Nestler, 2013; in: Park et al., 2021). The ventral tegmental area (VTA), the originating area of the dopaminergic mesocorticolimbic circuit, is involved in motivational stimuli processing and reward (Berridge, 2007; Nestler and Luscher, 2019; Lammel et al., 2014; Peña et al., 2017, 2019; Belujon and Grace, 2017). Interestingly, the VTA projections to brain regions such as amygdala, lateral septum, preFrontal Cortex, and nucleus accumbens have been implicated in bonding and attachment formation (Pedersen et al., 1994; Insel, 2003; Šešo et al., 2010; Curtis and Wang, 2005; Feldman, 2017; McCormick et al., 2019; Rincón-Cortés and Grace, 2020), and clinical and preclinical functional data indicate that experiences during early life affect the activity of neuronal pathways formed by VTA inducing behavioral long lasting consequences (Chocyk et al., 2011, 2015; Masrouri et al., 2020; Park et al., 2021; D'Addario et al., 2021; Oh et al., 2021).

Newsworthy, evidence suggest sex-dependent structural and functional differences in VTA dopaminergic system as well as sexually dimorphic response of midbrain to stress (Chocyk et al., 2015; Gillies et al., 2014; Rincón-Cortés and Grace, 2017; Kokane and Perrotti, 2020), and, intriguingly, several studies provide evidence for sex differences in many behaviors, as emotional, social, reward-associated behaviors and motivation (Brivio et al., 2020; Bath, 2020), all of them at least partially depending on brain networks linked to VTA (Berridge, 2007; Nestler and Carlezon, 2006; Koob and Volkow, 2010). Accordingly, many early stress-induced psychopathologies related to this brain system are reported to have a higher incidence in females than males (Keller and Roth, 2016; Seney and Logan, 2021; Bale, 2006).

Epigenetic modifications (Alyamani and Murgatroyd, 2018) are increasingly being recognized as critical to understand sex differences in brain development and response to early environment (Keller and Roth, 2016). A recent study showed that stress exposure during sensitive period alters in a sex dependent manner the transcriptional patterning in the VTA of mice modulating the susceptibility to adult stressful stimuli (Peña et al., 2019). However, due to methodological constrains (different adult stress, tissue processing), potential sex differences in response to stress exposure were not directly evaluated in male and female mice (Peña et al., 2019).

To our knowledge no study has directly investigated, to date, sex differences in VTA transcriptional patterning of mice exposed to early adversity. In addition, no study reported a stronger effect of early adversities affecting the attachment bond on VTA-related behavioral and transcriptomic pattern in female than male rodents, thus mirroring the consequences of early exposure to negative experiences commonly reported in human.

Based on these premises, in this study we reasoned that interfering with the formation of the attachment bond during early life could affect the VTA and some behaviors related to this critical brain area in a sex-dependent manner.

To test this hypothesis, we exploited our animal model of early adversity (Repeated Cross Fostering, RCF), which is hypothesized to interfere with the development of maternal attachment bond (D'Amato et al., 2011; Ventura et al., 2013; Luchetti et al., 2015, 2016; Di Segni et al., 2016, 2017, 2018, 2019, 2020; Dulor Finkler et al., 2020). We analyzed the long-lasting effect of RCF on behavioral and transcriptional profiles related to the VTA, across different strains in both sexes.

In order to investigate the behavioral consequences that may mostly reflect VTA-dysregulation, we took advantage of the Research Domain Criteria (RDoC) guidelines. The RDoC initiative (https://www.nimh.nih.gov/research/research-funded-by-nimh/rdoc/index.shtml) represents an attempt to create an experimental classification system where symptoms/domains, rather than mental disorders, are classified by grounding them in evidence based on etiology and pathophysiology (Insel, 2014; Anderzhanova et al., 2017; Söderlund and Lindskog, 2018).

Following these criteria, the behavioral domains that partially involve the VTA span across the positive and negative valence systems. We thus interpret behavioral data that were produced throughout the years in our laboratory and collected new behavioral data following RCF manipulation within this framework. We provide an overview wherein different behavioral constructs were evaluated based on their alteration – regardless of the directionality - with respect to control groups, in order to investigate a general level of "responsiveness" modulated by RCF experience in adult animals, rather than merely defining susceptibility or resilience to psychopathology. To examine the sex-dependent effects, the behavioral profile was investigated in both male and female mice. In addition, to strengthen our results, we extended our analysis across two different inbred strains of mouse, namely C57BL/6J (C57) and DBA/2J (DBA), known to respond very differently to early stress. This "across strains" analysis will help us identifying mechanisms that are conserved across different genetic background in females and males.

Finally, we relate the behavioral profile to transcriptional alterations induced by RCF in the VTA of adult male and female mice. Our comprehensive analysis of the VTA will evaluate if genetic sex drives different transcriptional profiles of the adult VTA, to what extent these differences can be generalized, and if sex-dependent developmental trajectories of the mesocorticolimbic dopaminergic system could be differently impacted by the disruption of the attachment bond, resulting in sex-specific behavioral alterations in adulthood.

## 2. Methods and materials

### 2.1. Animals

C57BL/6J (C57) and DBA2/J (DBA) female and male mice (Charles River Laboratories, Italy) were housed with water and food available ad libitum, at constant room temperature (21 ± 1 °C) and in a 12:12 h light–dark cycle (lights on at 07:00 a.m.). Adequate measures were taken to minimize pain or discomfort of mice and all experiments were carried out in accordance with Italian national law (DL 116/92 and DL 26/2014) on the use of animals for research based on the European Communities Council Directives (86/609/EEC and 2010/63/UE). Experimental protocol (no. 769/2017) was approved by Italian Ministry of Health. Mice 10–12 weeks old were used for both behavioral and RNA-Seq experiments, if not differently reported.

### 2.2. Repeated Cross Fostering (RCF)

RCF was performed as previously described (D'Amato et al., 2011; Ventura et al., 2013; Luchetti et al., 2015, 2016; Di Segni et al., 2016, 2019, 2020; D'Addario et al., 2021). Pups from the same litter spent the first postnatal day (PND0) with their biological mother. On PND1, litters were randomly assigned to experimental (RCF) or Control (CTR) group. RCF pups were fostered by moving the entire litter into the home cage of a different mother, whose pups had just been moved to an adoptive mother. This procedure was repeated daily (since PND1 until PND4); on PND4 pups were left with the last adoptive mother until weaning. CTR litters were only picked up daily and reintroduced in their home cage; this procedure was carried out within 30 s. Animals were weaned at PND28, separated by sex and housed in groups of 4 littermates. RCF and CTR groups were sorted by collecting max 2 individuals per cage/litter (Ventura et al., 2013; Di Segni et al., 2016, 2017, 2019) in order to avoid litter effects. Different animals were used for RNA Sequencing and behavioral experiments.

### 2.3. Pups' attachment behavior

Attachment behavior was measured in pups by ultrasonic

vocalizations (USVs) during separation from the mother, and by homing test, that is measuring motivation to orient towards their mother's cues (D'Amato et al., 2011; Luchetti et al., 2021; Cinque et al., 2012).

*USVs*: USVs were measured at PND8 as previously described (Cinque et al., 2012; Luchetti et al., 2021). No more than 4 pups x litter were tested: each pup was individually placed into a beaker containing (i) home-cage bedding (USVs-Home-Cage) or (ii) clean bedding (USVs-Clean) and vocalizations were recorded during a 5 min session. Ultrasonic vocalizations were recorded and analyzed thanks to dedicated software (Avisoft Bioacoustics, Berlin, Germany). Sample size for each experimental group was 8–10 and pups from each group belonged from 5 to 6 different litters (D'Amato et al., 2011).

*Homing*: The Homing test was performed at PND10 as previously reported (Cinque et al., 2012; Luchetti et al., 2021). The ability of pups to orient towards familiar odorous cues was evaluated in a small apparatus (5 × 33 × 10 h cm) with a central Plexiglas part (5 × 5 cm, starting point) that separated (with sliding doors) two differently scented arms. One arm was covered with pup's home-cage bedding, whilst the other one was covered with clean bedding. The pups were left 45–60 s in the central part of the apparatus before opening doors and allowing them to move freely for 5 min. The behavior of the pup was video-recorded and the time spent in the different arms of the apparatus was evaluated thereafter by video-tracking software (SMART 1.1, Panlab). The apparatus was carefully cleaned with 10% ethanol solution at the end of each test session. No more than 4 pups per litter were tested.

### 2.4. Adult behavioral tests

Animals from different cohorts were used for behavioral tests; however, the same cohort was always used to directly compare male and females mice.

#### 2.4.1. Saccharin preference test (SPT)

The saccharin preference test was carried out as previously described (Di Segni et al., 2016, 2019). During the habituation day, mice were singly moved in a cage with two bottle containing water; after 24h they were exposed to a double choice drinking test (saccharin solution [0.5%] or drinking water) from graduated tubes (10 ml volume). Intake was measured to the nearest 0.1 ml. The test cage was the same throughout the experiment. Only mice drinking at least 0.1 ml on day 1 were included in the study. The percentage of saccharin intake (saccharin intake (ml) *100/saccharin + H20 intake (ml)) was evaluated.

#### 2.4.2. Forced swim test (FST)

Mice were individually placed in an 18 cm-diameter glass cylinder (height 40 cm) filled with 20 cm of water at 28 ± 2 °C as previously described (Ventura et al., 2013; Di Segni et al., 2016, 2019). Behavioral response was video-recorded for 10 min using a digital camera placed in the front of the apparatus before returning the mice to the home cage. The duration (seconds) of immobility was taken as dependent variable and manually scored with "EthoVision" (Noldus, The Netherlands) software by a trained observer blind to the animals' treatment.

#### 2.4.3. Tail suspension test (TST)

Each mouse was suspended by the tail at 60 cm above the floor in a white plastic chamber using adhesive tape placed <1 cm from the tip of the tail according to Yan et al. (2015). Behavior was recorded for 10 min using a digital camera placed in the front of the apparatus. Duration of immobility was manually scored with "EthoVision" (Noldus, The Netherlands) by a trained observer blind to the animals' treatment. In this test, the immobility was defined as the period when the animals stopped struggling for ≥1 s.

#### 2.4.4. Social interaction test (SIT)

Different groups of animals were tested on PND28 and PND60 in a gray plexiglas rectangular box (60 × 40 × 24 cm) consisting of an empty central "starting chamber" interconnected with two "stimulus chambers" where two identical clear plexiglas cylinders (8 cm in diameter) with multiple small holes were placed (Cinque et al., 2012; Fiori et al., 2015). During the habituation session (10 min) the mouse was placed in the central chamber and allowed to freely explore the whole apparatus. An age- and sex-matched mouse was then introduced as social stimulus into one cylinder (pseudo-randomly chosen) whereas an object was introduced into the other, for the 10 min test session. Both sessions were recorded and the time the subject mouse spent in each chamber was measured by a video-tracking system (Smart 1.1). Time spent sniffing each cylinder in the test session was manually scored by a blind trained observer to calculate the sociability index: time spent sniffing cylinder with social stimulus x 100/time spent sniffing both cylinders (Fiori et al., 2015).

#### 2.4.5. Elevated plus maze test (EPM)

Mice were individually tested in a 5 min session in an apparatus elevated of 38.5 cm above the floor and consisting in two open arms (27 × 5 cm) and two closed arms (27 × 5 × 15 cm) connected by a central platform (5 × 5 cm) representing the starting point (Di Segni et al. 2016, 2019). The percentage of entries in the open arms (open entries/open + closed x 100), and the percentage of time spent in the open arms (time in open/open + closed x 100) were collected and analyzed by the "EthoVision" (Noldus, The Netherlands) fully automated video tracking system.

#### 2.4.6. Open field test (OFT)

The mouse was introduced in the central sector and left to explore the apparatus consisting in a circular open field, 60 cm in diameter and 20 cm in height for 5 min (Di Segni et al., 2016, 2019). Distance moved (cm), was video-recorded and analyzed by the "EthoVision" (Noldus, The Netherlands) fully automated video tracking system.

### 2.5. Tissue isolation and RNA preparation

RCF and control (CTR) animals from the same cohort were used for this experiment. RCF-treated and CTR female and male mice from DBA and C57 strains (n = 5 per experimental group) were sacrificed by cervical dislocation at PND60. Brains were dissected and stored at – 80 °C. Bilateral punches of VTA were obtained from coronal brain slices (Peña et al., 2019) no thicker than 300 μm using a stainless-steel tube of 0.5 mm inside diameter and stored at T = – 80 °C until the day of the assay. The coordinates were measured according to the atlas of Paxinos and Franklin (2019). A schematic representation of VTA punch is provided in Supplementary Fig. 1. RNA from punches was subsequently isolated using Total RNA purification Plus Kit (Norgen Biotek, Thorold, Canada). RNA quantity was determined by absorbance at 260 nm using a NanoDrop UV-VIS spectrophotometer.

#### 2.5.1. RNA library preparation and sequencing

mRNA sequencing was performed at Iga technology service (Udine). RNA from individual VTA samples (about 200 ng each) was quantified and quality tested by Agilent 2100 Bioanalyzer RNA assay (Agilent technologies, Santa Clara, CA) or Caliper (PerkinElmer, Waltham, MA). Only RNA samples with RIN value > 7.0 were included in the study.

Universal Plus mRNA-Seq kit (Tecan Genomics, Redwood City, CA) has been used for library preparation following the manufacturer's instructions (library type: fr-secondstrand). Final libraries were checked with both Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and Agilent Bioanalyzer DNA assay or Caliper (PerkinElmer, Waltham, MA).

Libraries were then prepared for sequencing and sequenced on single-end 75 bp mode on NextSeq 500 (Illumina, San Diego, CA). The number of reads (in millions) produced for each sample ranged between 25 and 35.

Primary bioinformatic analysis included: a) processing raw data for

both format conversion and de-multiplexing by Bcl2Fastq 2.20 version of the Illumina pipeline (https://support.illumina.com/content/dam/illumina-support/documents/documentation/software_documentation/bcl2fastq/bcl2fastq2-v2-20-software-guide-15051736-03.pdf); b) Adapter sequences masking with Cutadapt v1.11 from raw fastq data using the following parameters: -anywhere (on both adapter sequences) –overlap 5 –times 2 –minimum-length 35 –mask-adapter (Martin, 2011).

The RNA-seq dataset is available from the GEO database under the accession number GSE165922 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE165922).

#### 2.5.2. mRNA-seq data analysis

Color Space sequence reads were mapped against the most recent version of the mouse reference genome (GRCm38/mm10) from UCSC, using RefSeq as the gene structure and annotation database. The software used for mapping and quantification of transcripts was Lifetech Lifescope version 2.5.1. Quality control of the aligned reads was performed with the FastQC software.

Read counts were extracted from Lifescope mapping results with proprietary software, samples showing as outliers in a PCA (highlighted in Supplementary Figs. 2 and 3) were excluded from the subsequent analyses. Specifically, samples that, by visual inspection in the PCA, appeared distant from the whole group of experimental mice (C57, male or female and DBA, male or female) regardless the treatment were excluded (Supplementary Fig. 2); only one sample (Ctrl C57-F mouse) was removed to reduce variance and allow the analysis of more consistently separated groups.

Samples analyzed were respectively: for DBA: RCF male, n = 5 RCF female, n = 5, CTR male, n = 4, CTR female, n = 4; for C57: RCF male, n = 5, RCF female, n = 4, CTR male, n = 3, CTR female, n = 4. The differential expression analysis was performed with the Bioconductor DeSeq2 library (Love et al., 2014). For our analyses, we selected those genes whose fold changes were statistically different according to the FDR-corrected $p < 0.05$. The interaction between the effect of "Strain" and "RCF-treatment" on the expression of individual genes was assayed by two-way ANOVA ($p < 0.05$ for significance).

Chromosome locations were extracted from BioMart (Durinck et al., 2009) and analyzed with in-house scripts, pathway enrichments were performed with David (Huang et al., 2009).

To compare pairs of differential analysis (C57 RCF females vs Control females compared to DBA RCF females vs Control females; C57 CTR females vs. C57 CTR males compared to C57 RCF females vs. C57 CTR females; DBA CTR females vs. DBA CTR males compared to DBA RCF females vs. DBA CTR females) we used the Rank-Rank hypergeometric overlap test (Oldham Green et al., 2021; Peña et al., 2019) using the R package (Rosenblatt and Stein, 2014). The Rank-Rank scatter plots comparing pairs of gene lists were generated by applying the RRHO function provided by the R package on threshold-free lists in which genes were ranked by the negative log10 of the adjusted p-value multiplied by the sign of the fold change as determined by DESeq2.

Male and female VTA tissues sequenced in this study were from the same cohort, making possible a direct comparison of the samples.

### 2.6. Statistics

Two-way ANOVA (condition, 2 levels: RCF, CTR; bedding, 2 levels: Clean, Home-cage) was used to evaluate differences in pups' vocalizations (USVs) for each strain. Three-way ANOVA for repeated measures (sex, 2 levels: males, females; condition, 2 levels: RCF, CTR; bedding, 2 levels: Clean, Home-cage) was used to evaluate pups' Homing test results for each strain. One sample Student's *t* -test was performed for the time spent in immobility during FST, for percentage of time spent in the open arms during EPM and for distance moved during OFT for DBA male mice (RCF and CTR group). Moreover, one sample Student's *t* -test was performed for time spent in immobility during TST and for sociability index during SIP (PND28 and PND60) for each strain and sex.

The responsivity index was calculated by attributing a binary value (1/0: different/no-different response between RCF and CTR mice within each group, represented with "*" or "ns" respectively into the table) to the results of each test per group and evaluating the percentage of responsivity for each group on the sum of significant responses throughout all groups (total responsivity). [e.g. C57 RCF female mice responsivity is: EPM (0) + OFT (0) + FST (1) + TST (0) + SIT P20 (1) + SIT P60(0) + SPT (1) + CPP 2.5 mg (1) + CPP 5 mg (0) = 4; C57 RCF female general responsivity index is the percentage of C57 RCF female responsivity on the sum of total responsivity: (4/(4 + 2+4 + 0))*100 = 40%].

Standard hypergeometric overlap test was carried out using the phyper R function (Oldham Green et al., 2021; Peña et al., 2019).

## 3. Results

### 3.1. RCF interferes with the attachment bond formation

To test if the RCF procedure (Fig. 1A) alters the formation of the attachment bond, we evaluated the emission of USVs during separation from the mother and we performed the homing test in CTR and RCF groups (Fig. 1B).

ANOVA for USVs showed a significant condition × bedding interaction for both strains (C57: $F(1,33) = 8.04$; $p < 0.01$; DBA: $F(1,27) = 6.34$; $p < 0.05$). Both C57 and DBA pups exposed to RCF differed from their respective CTR for the amount of ultrasonic calls emitted (Fig. 1C and D). CTR pups of both strains vocalized less in home-cage bedding than in the clean, odorless bedding (C57: $p < 0.05$; DBA: $p < 0.05$), thus confirming the calming effects of home cage bedding on pups' calls. However, this effect was not evident in RCF mice (both strains) that appeared unable to benefit from the familiar home-cage cues (Fig. 1C and D).

Concerning the Homing test, three-way ANOVA showed a significant sex x condition × bedding interaction for both strains (C57: $F(1,28) = 5.80$; $p < 0.05$; DBA: $F(1,31) = 5.56$; $p < 0.05$). The results confirmed that all pups prefer the familiar (mother) scented part of the apparatus, spending the most part of the time there (Fig. 1E–H). However, RCF females spent significantly more time in the home-cage (the environment providing information about proximity to mother) in comparison with CTR females, regardless of the genotype ($p < 0.05$) (Fig. 1 E, G).

### 3.2. RCF affects adult VTA-related behavioral domains in a sex-dependent manner

We collected results from several behavioral experiments to assay the general impact of RCF on adult VTA-related behavioral domains (according to the RDoC) in two different inbred strains of both sexes. The behavioral tests examined included FST, EPM, OFT, TST measuring the responses to (potential and acute) threatening stimuli and thus the functionality of the negative valence system (Fig. 2A); the SIT, SPT and cocaine-induced CPP, measuring the responses to rewarding stimuli (reward evaluation and learning) and thus the functionality of the positive valence system (Fig. 2A). Results were collected from previous (Di Segni et al., 2016, 2017, 2019, 2020; D'Addario et al., 2021) and new experiments (see Methods section for details). Significant modifications induced by RCF on any of these behaviors were signed with an asterisk in the Fig. 2A (mean and standard errors are reported in the Supplementary Fig. 4).

Specifically, no significant difference was evident between RCF and CTR DBA males in FST ($t = -.475$; p = ns). Moreover, RCF did not induce significant differences in DBA male mice in EPM ($t = -0.746$; p = ns) and OFT (moved distance: $t = -0.633$; p = ns; percentage of time spent in external arena: $t = -1.256$; p = ns) and SPT ($t = -0.241$; p ns) (Fig. 2A). Concerning TST, no significant difference was observed between RCF and CTR DBA males ($t = .689$; p = ns), between RCF and CTR C57 males ($t = 0.183$; p = ns), and females ($t = 1.022$; p = ns) (Fig. 2A).

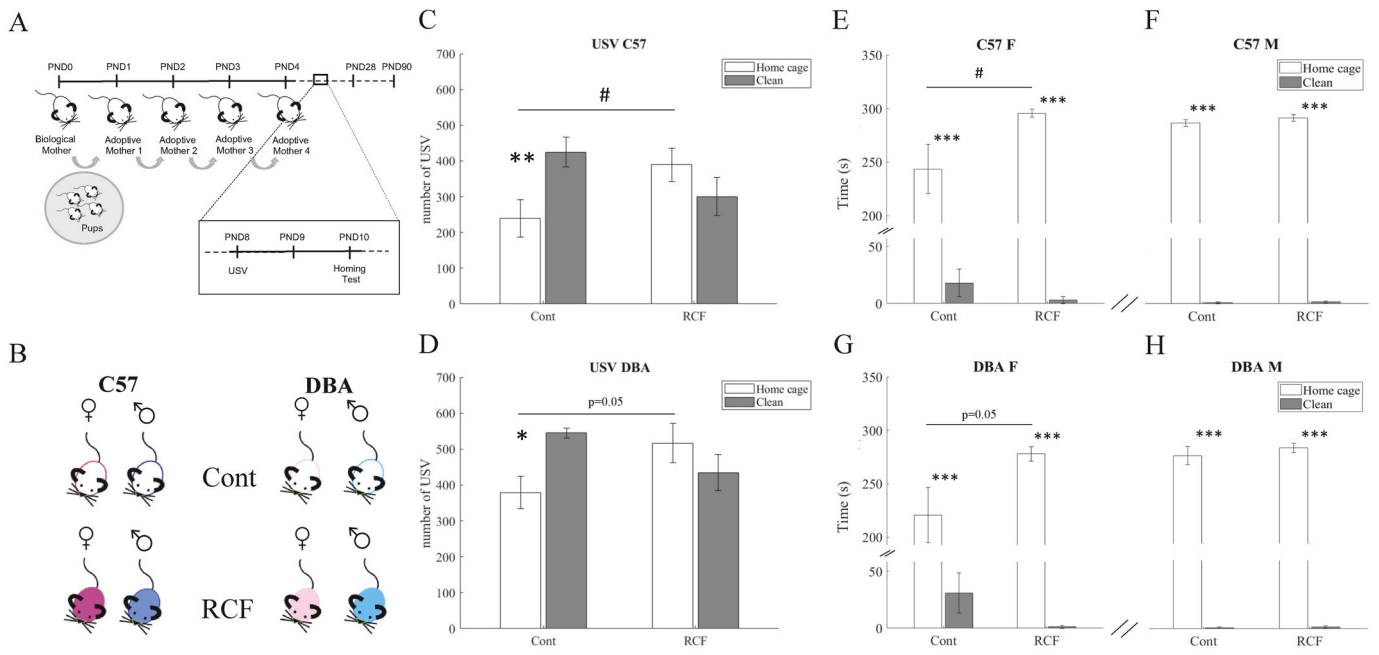



**Fig. 1. A.** Schematic representation of Repeated Cross Fostering (RCF) procedure and timeline of pups behavior. RCF pups change adoptive mother four time from post-natal day (PND) 1 until PND4; Control pups are removed from and reintroduced in the home cage for the same days. **B.** Schematic representation of experimental groups: RCF and Cont male and female mice from C57 and DBA strains. **C,D.** USVs emitted by from C57 (**C**) and DBA (**D**) pups on PND8 in the Home cage and in the Clean cage. *p < 0.05; **p < 0.01. E. Time spent in the Home cage and in the clean cage during the Homing test (PND10) by RCF and Cont male and female mice from C57 (**E,F**) and DBA (**G,H**) pups. ***p < 0.001; # <0.05.

A

VTA-related RDoc domains:

| Domain | Construct | test | C57 F | C57 M | DBA F | DBA M | References |
|---|---|---|---|---|---|---|---|
| Negative Valence System | Potential Threat | EPM | ns | ns | ns | ns | Di Segni et al., 2016; Di Segni et al., 2019; Results section |
| | Acute Threat | OFT | ns | ns | ns | ns | Di Segni et al., 2016; Di Segni et al., 2019; see results |
| | | FST | * | * | * | ns | Di Segni et al., 2016; Di Segni et al., 2019; Results section |
| | | TST | ns | ns | * | ns | Results section |
| Positive Valence System | Reward Valuation | SIT P28 | * | ns | ns | ns | Results section |
| | | SIT P90 | ns | ns | ns | ns | Results section |
| | | SPT | * | * | * | ns | Di Segni et al., 2016; Di Segni et al., 2019; Results section |
| | Reward Learning | CPP Cocaine 2,5/5 mg | * | ns | ns | *Missing results* | Di Segni et al., 2017; Di Segni et al., 2019 |
| | | CPP Cocaine 5/7,5 mg | ns | ns | * | *Missing results* | Di Segni et al., 2017; Di Segni et al., 2019 |

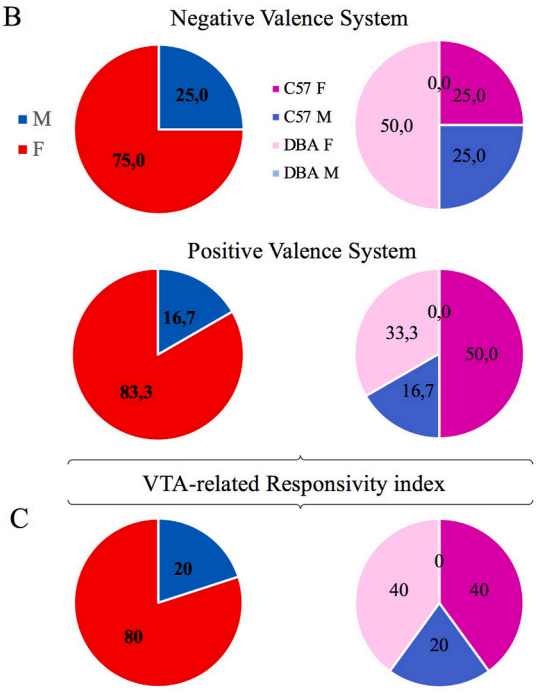



**Fig. 2. A.** Summary of behavioral tests carried out in RCF male and female mice from C57 and DBA strains in comparison with the respective control groups. *: significantly different compared to the respective control groups; ns: no significant; EPM: elevated plus maze; OFT: open field test; FST: forced swimming test; TST: tail suspension test; SIT: social interaction test; SPT: saccharin preference test; CPP: conditioned place preference. **B.** Grouped data for males and females from both genotypes for Negative Valence System and Positive Valence System. **C.** VTA-related Responsivity index (calculated as reported in the results section).

Finally, a significant difference was evident between RCF and CTR DBA females ($t = -2.571$; $p < 0.05$) with increased immobility in RCF compared to CTR group (Fig. 2A).

A significant effect of RCF on sociability at PND28 was evident only in C57 females (C57 females: $t = -2.4$; $p < 0.05$; C57 males: $t = -0.45$; $p =$ ns; DBA females: $t = -0.24$; $p =$ ns; DBA males: $t = -0.13$; $p =$ ns) with increased sociability in RCF in comparison with CTR group (Fig. 2A). No significant effect of RCF was evident at PND60 regardless of strain and sex (C57 females: $t = 0.06$; $p =$ ns; C57 males: $t = -0.001$; $p =$ ns; DBA females: $t = -0.32$; $p =$ ns; DBA males: $t = -0.79$; $p =$ ns)

(Fig. 2A). To offer a quantitative glance to the overall behavioral responses that have examined, we built-up a VTA-related "responsivity index" that is reported in pie charts (Fig. 2B and C).

Based on this global view, our results indicate that RCF manipulation induces long lasting behavioral effects more strongly affecting female than male mice, regardless of the genotype (Fig. 2B). In addition, by breaking up data for strain and sex, results indicate that RCF C57 females explain ~40% of effects related to positive stimuli response while RCF DBA females account for ~40% of effects related to negative stimuli response, according with the increased sensitivity to rewarding stimuli effects and vulnerability to depression-like phenotype reported, respectively, in adult RCF C57 and DBA female mice (Di Segni et al., 2016; 201). Only a limited effect of RCF is evident in C57 males (~20%) and no significant impact is observed in DBA males (Fig. 2B).

### 3.3. RCF impacts adult transcriptional profile of VTA almost exclusively in females

Through next-generation mRNA-Seq, we profiled VTA transcriptomes in RCF and CTR adult male and female mice from C57 and DBA backgrounds.

First, we compared the expression levels of VTA genes between RCF and CTR mice in males and females within a specific background (Fig. 3A). We found that the number of differentially expressed genes (DEGs) in RCF versus CTR females was tremendously higher than the number of DEGs in male, and this was true for both genotypes (Fig. 3A). Specifically, in C57 mice RCF altered the expression of 18 genes in males (n = 7 UP and n = 11 DOWN) and 1593 in females (n = 900 UP and n = 693 DOWN). Similarly, in DBA mice we found only 2 DEGs in RCF males (all UP) while 1090 DEGs were found in females (n = 608 UP and n = 482 DOWN). Moreover, we did not observe specific chromosomal enrichment of DEGs in any of the two strains, when they were distributed according to their location on different chromosomes (Fig. 3B).

A list of DEGs for male and female for each strain is presented in the supplementary Tables (Supplementary Tables 1, 2, 3 and 4). Notably, by examining the DEGs induced by RCF in female mice from the two genetic backgrounds we found 75 overlapping genes, that are shown in the heatmap (Fig. 3C, listed in Supplementary Fig. 5 and marked with an asterisk in the Supplementary Tables 1 and 2). Interestingly, 32 genes out of the 75 overlapping display a discordant sign of fold changes between the two genotypes (and significant treatment × genotype interaction in a two-Way ANOVA), likely representing opposite adaptations to the RCF in the two different genotypes (Supplementary Fig. 5). However, we did not observe a significant overlap between the DEGs from the two lists (DEGs between RCF vs Control C57 females and DEGs between RCF vs Control DBA females) using the hypergeometric overlap test.

To describe the physiological functions that are mostly represented among the altered transcripts in female mice, we classified them by Gene Ontology analysis, using the DAVID web-based tool [http://david.abcc.ncifcrf.gov/] for the category of "KEGG pathways". We found n = 24 significant pathways for C57 mice, n = 10 for DBA and n = 4 overlapping pathways between the two strains (Fig. 4A). Specifically, the DEGs in the VTA of C57 females were significantly enriched for genes that are mainly involved in synaptic function, including the dopaminergic, glutamatergic, and GABAergic synapse, in signal transduction processes, including Calcium signaling, cAMP, Rap1, or P13K-Akt signaling pathways and several extracellular matrix-related functions. Notably, biological processes involved in drug addiction (morphine, cocaine, nicotine and amphetamine addiction) were also significantly enriched of DEGs in C57 mice (Fig. 4B). In parallel, DEGs in DBA females were significantly enriched for genes that are mainly involved in Axon guidance, Focal adhesion, P13K-Akt signaling pathways, Oxidative phosphorylation, ECM-related functions and neurodegenerative diseases (including Alzheimer, Parkinson and Huntington disease) (Fig. 4B). Common pathways to two strains are detailed in the Fig. 4B.

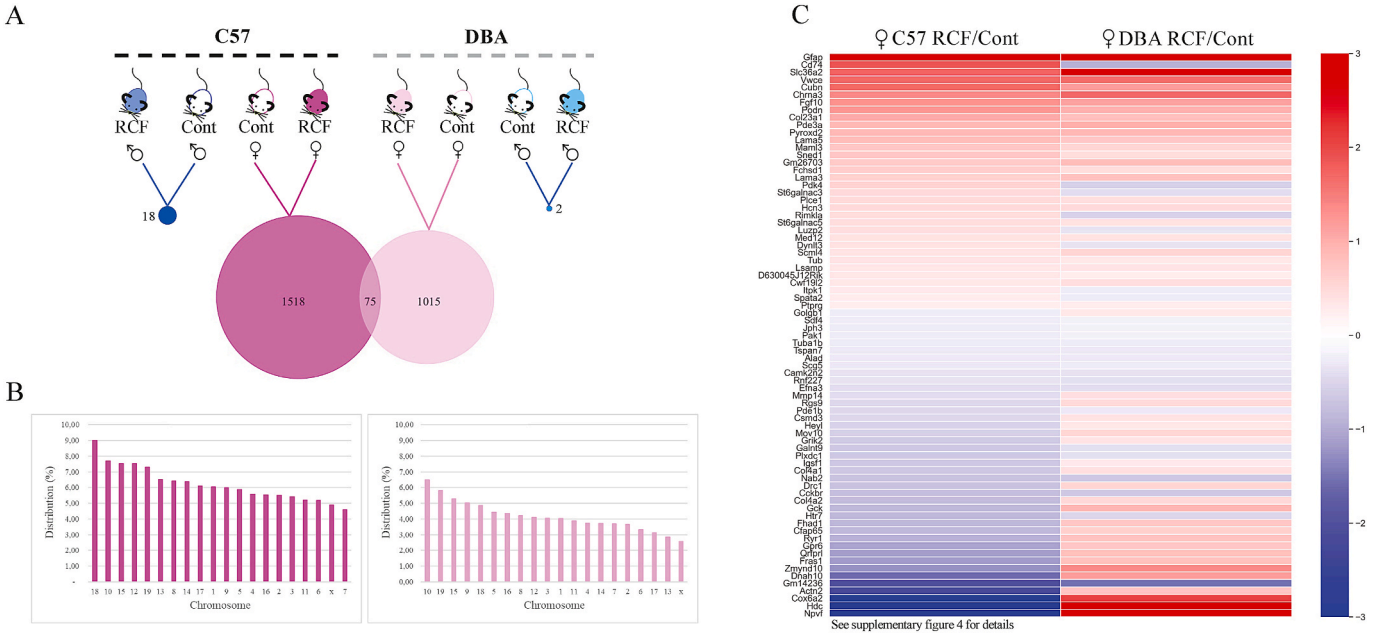
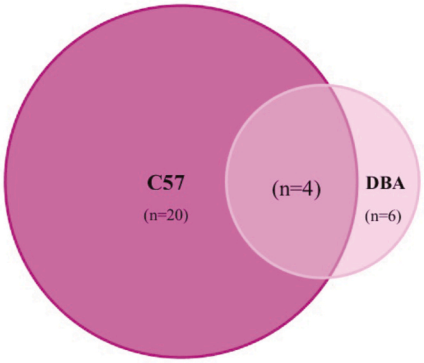



**Fig. 3. A.** Schematic representation of differential analysis of VTA transcriptomes between male and female RCF and control mice across different strains (C57 and DBA). Venn diagrams indicate the number of differentially expressed genes (DEGs) between each pair of experimental groups. The colors blue and pink of the illustrated mice represent males and females, respectively. **B.** Histograms represent the chromosomal distribution of the DEGs between RCF versus control female mice from C57 (left histogram) and DBA (right histogram) strains. The number of DEGs per chromosome is expressed as percentage over the total coding genes (including pseudogenes) located in the chromosome. **C.** DEGs that are altered in RCF female mice common to both strains (n = 75) are shown in the heatmap. Color intensity is proportional to the log2FC calculated between the RCF and Control group per each strain. Specifically, red and blue colors indicate genes that are upregulated and downregulated in RCF versus control mice, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

A




B

| C57BL/6J pathways | DBA/2J pathways | Common pathways With Benjamini Correction ≤ 0,05: |
|---|---|---|
| - cAMP signaling pathway<br>- Neuroactive ligand-receptor interaction<br>- Pathways in cancer<br>- Morphine addiction<br>- Calcium signaling pathway<br>- Glutamatergic synapse<br>- Circadian entrainment<br>- **Focal adhesion**<br>- Nicotine addiction<br>- Rap1 signaling pathway<br>- Cocaine addiction<br>- Retrograde endocannabinoid signaling<br>- **ECM-receptor interaction**<br>- GABAergic synapse<br>- Regulation of actin cytoskeleton<br>- Dopaminergic synapse<br>- Ras signaling pathway<br>- **Axon guidance**<br>- **PI3K-Akt signaling pathway**<br>- Proteoglycans in cancer<br>- Transcriptional misregulation in cancer<br>- Amphetamine addiction<br>- Melanoma<br>- MAPK signaling pathway | - **ECM-receptor interaction**<br>- Oxidative phosphorylation<br>- Parkinson's disease<br>- Alzheimer's disease<br>- Huntington's disease<br>- **Focal adhesion**<br>- **Axon guidance**<br>- Non-alcoholic fatty liver disease (NAFLD)<br>- **PI3K-Akt signaling pathway**<br>- Metabolic pathways | - **Focal adhesion**<br>- **ECM-receptor interaction**<br>- **Axon guidance**<br>- **PI3K-Akt signaling pathway** |

**Fig. 4. A.** The Venn diagram indicates the number of "KEGG pathways" that are significantly enriched in the DEGs between RCF and control mice in C57 (dark pink) or DBA (light pink) female mice. Number of common pathways are indicated in the intersection. **B.** The pathways included in the Venn diagram are specified in the table. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

### *3.4. Sex-specific VTA transcriptional profiles reveal female-specific genes that are sensitive to RCF manipulation*

Based on the greater effect of RCF in female than male mice, we hypothesized that transcriptional changes induced by RCF in female mice could involve indeed sex-specific VTA genes differently expressed between CTR males and females.

To evaluate this hypothesis, we first compared the expression level of VTA genes between females and males in CTR mice within each background. We found three hundred and one (301, of which 174 Down and 127 Up regulated in females) and one hundred and forty-four (144, of which 116 Down and 28 Up regulated in females) genes that were differently expressed between females and males in C57 and DBA strains, respectively (Fig. 5A and Supplementary Tables 5 and 6). Nine overlapping genes were found between the two strains' lists, mainly genes located on sex chromosomes. Gene Ontology analysis for each list is reported in Supplementary Table 7.

When we clustered these genes according to their chromosomal location, we did not observe specific chromosomal enrichment (Fig. 5B). Following, we matched these lists of DEGs, with DEGs that were obtained comparing RCF vs CTR females within each strain. Interestingly, we found an overlap of ~50% of genes for both strains (n = 167 genes for C57 (55%); n = 82 genes for DBA (57%)) (Fig. 6A–C and Supplementary Tables 8 and 9). This overlap was highly significant as determined by the hypergeometric test (C57 CTR females vs. C57 CTR males compared to C57 RCF females vs. C57 CTR females: $p < 0.0001$; DBA CTR females vs. DBA CTR males compared to DBA RCF females vs. DBA CTR females: $p < 0.0001$). Interestingly, the Rank-Rank hypergeometric overlap test revealed a striking discordance in the direction of changes, wherein the RCF manipulation in female mice induces transcriptional changes in opposite direction with respect to their difference versus male mice. This anti-correlation is illustrated by the scatter plots in Fig. 6 D, E and Supplementary Fig. 6).

## 4. Discussion

In this work we report, for the first time, that an early experience that affect the attachment bond, impacts in the long-term VTA-related behavioral and transcriptomic pattern almost exclusively in female mice. Notably, these effects seem to be genotype-independent, since they are conserved in two different inbred strains, thus improving the translational value of our results. A stronger impact of early adversities is indeed reported in women rather than men (Hodes and Epperson, 2019; Pratchett et al., 2010; Weiss et al., 1999).

First, we evaluated if our model of early adversity interferes with the attachment bond formation in a sex-related manner. The distress vocalizations are commonly used to study attachment behavior in puppies (Moles et al., 2004; Luchetti et al., 2015, 2016). In the RCF condition, the repeated change of caregiver is aimed to interfering with the formation of a stable and predictable infant-mother bond. Control pups vocalized more consistently in absence of familiar cues (Clean condition), indicating that the home cage bedding reduced the emotional impact of social isolation (Cinque et al., 2012). However, the consolatory/calming effect of the home cage bedding (representing the mother) was not evident in RCF pups from both strains. In fact, according to prediction, RCF mice from both strains emitted at PND8 a higher number of separation-induced ultrasonic vocalizations (USVS) selectively when exposed to olfactory cues that provide information about proximity to mother (home-cage *vs* clean bedding). Our results confirm and expand previous results obtained in outbred and inbred mouse as well as in rat showing that RCF impairs the formation of attachment bond (D'Amato et al., 2011; Ventura et al., 2013; Di Segni et al., 2018; Dulor Finkler et al., 2020). Interestingly, when tested in the Homing Test at PND10, although all groups showed preference for the home-cage compared to the clean-cage, RCF females spent significantly more time in the home-cage (the environment providing information about proximity to mother) in comparison with Control females, regardless of the genotype. These data support increased separation anxiety induced by RCF manipulation due to compromised bond attachment formation. Unexpectedly, no difference was instead evident

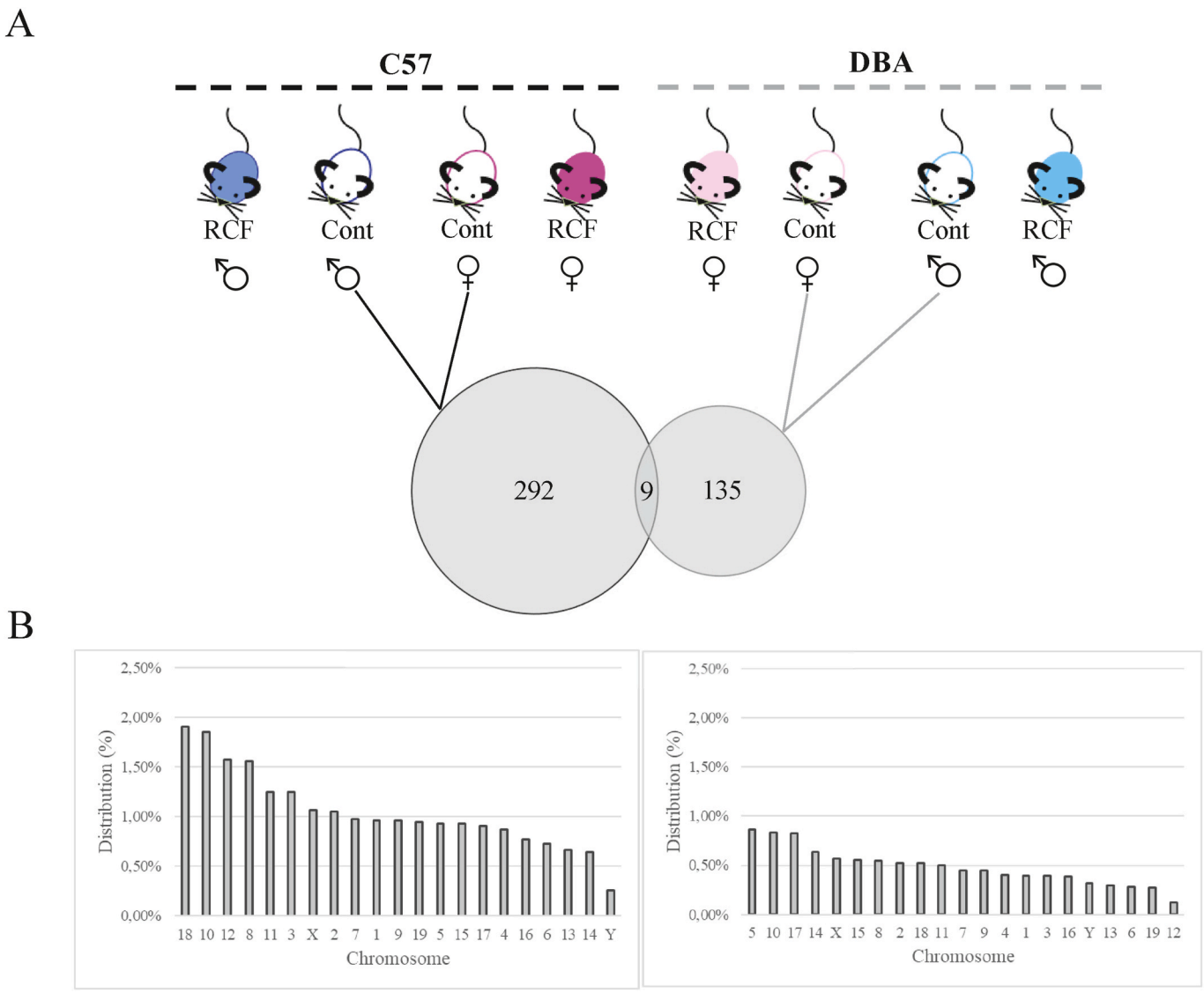



**Fig. 5. A.** Schematic representation of the differential analysis of VTA transcriptomes between male and female control mice within each strain (C57 and DBA). Venn diagrams indicate number of DEGs between each pair of experimental groups. **B.** Histograms represent the chromosomal distribution of the DEGs between male versus female control mice from C57 (left histogram) and DBA (right histogram) strains. The number of DEGs per chromosome is expressed as percentage over the total coding genes (including pseudogenes) located in the chromosome.

in males, suggesting that males were less affected by RCF manipulation. All together, these results confirm previous studies reporting early onset of sex-dependent differences induced by post-natal stress (Csikota et al., 2020) and indicate an early stronger effect of RCF in females compared to males. To note, cross-fostered pups were not neglected by adoptive mothers and did not differ from Control animals in terms of received maternal care (e.g. nursing, grooming/licking) as previously reported (D'Amato et al., 2011; Luchetti et al., 2015, 2016; Di Segni et al., 2016).

Based on temporal overlap between the sensitive period for the attachment bond (Opendak et al., 2017) and the development of dopaminergic system (Luo and Huang, 2016), as well as on previous reports suggesting that maternal attachment bond may also be linked to dopaminergic system (Strathearn et al., 2009; Curtis and Wang, 2005), here we speculate that our manipulation, carried out within the sensitive period for dopamine system development (Luo and Huang, 2016), could have altered VTA functions and compromised the attachment bond affecting, in turn, the response to future events in adulthood. Interestingly, the RCF effects were more evident in female than male pups.

Accordingly, our previous and present results indicate that RCF manipulation induces long lasting VTA-related behavioral effects (Ventura et al., 2013; Di Segni et al., 2016, 2017, 2020) more strongly affecting female than male mice (Di Segni et al., 2019), across two genotypes. In addition, RCF differently affects Positive and Negative Valence Systems in C57 and DBA females, respectively. In particular, consistently with previous and present data showing increased sensitivity to saccharine and cocaine (Di Segni et al., 2016, 2017) and increased sociability index at PND28, adult RCF C57 female mice show increased sensitivity to positive motivational stimuli (sum up in the Positive Valence System). Otherwise, according with increased passive coping strategy observed in our previous (Di Segni et al., 2016) and present results (see TST results section), RCF DBA female mice show increased sensitivity to aversive stimuli mediating acute threat response (sum up in the Negative Valence System).

Consistently with behavioral data, we found that RCF deeply altered VTA transcriptional profile almost exclusively in females and, again, this pattern was conserved among different mouse backgrounds. We found a tremendously higher number of DEGs in RCF females (1593 in RCF C57 females; 1090 in RCF DBA females), with small or no effect in RCF males (18 in RCF C57 males, 2 in RCF DBA males), again suggesting a higher plasticity in female VTA in response to maternal environment.

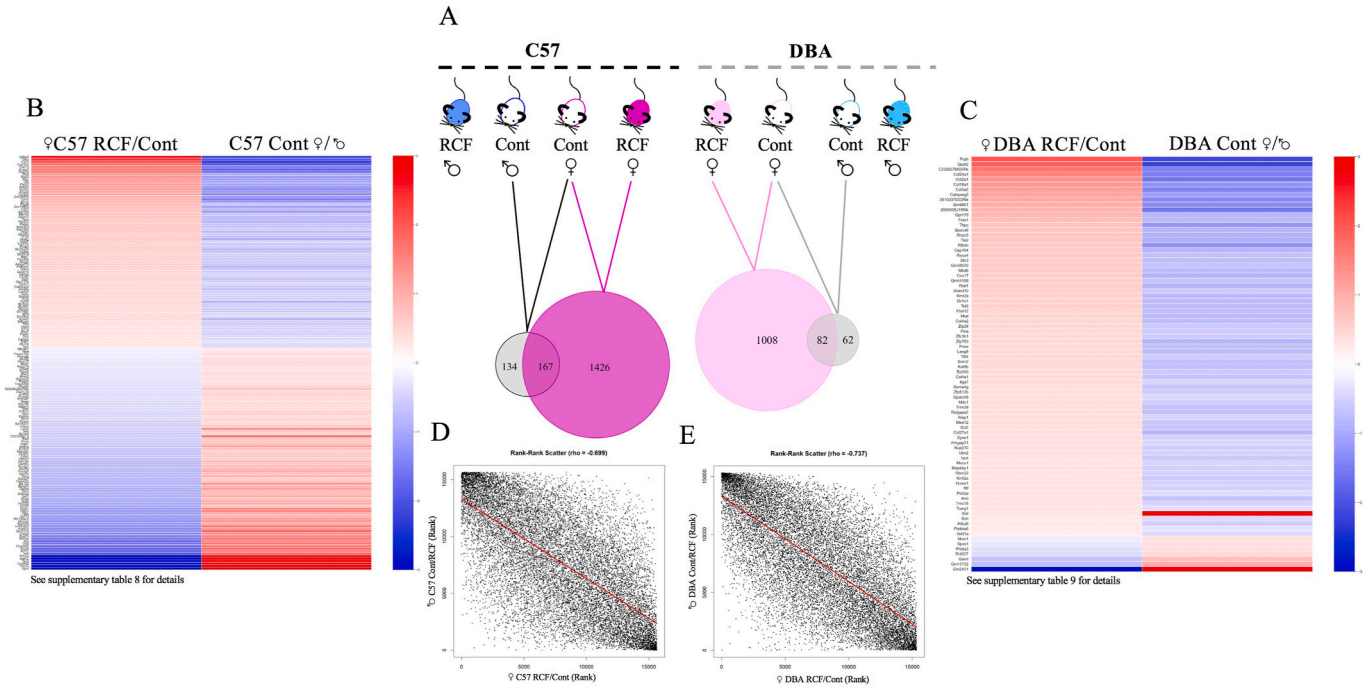



**Fig. 6. A.** Schematic representation of the differential analysis of VTA transcriptomes between male and female control mice, and RCF versus control female mice within each strain (C57, DBA). Venn diagrams indicate the number of DEGs between each pair of experimental groups. The number of overlapping DEGs is indicated in the inter section. **B-C.** DEGs indicated in the intersection for C57 (on the left) and for DBA (on the right) are shown in the heatmaps (C57: **B**; DBA: **C**). Color intensity is proportional to the log2FC calculated between RCF versus Control group or male versus female control group per each strain. Specifically, red colors indicate genes that are upregulated in RCF versus Control, or in male versus female mice, while blue colors indicate genes that are downregulated in RCF versus Control, or in male versus female group. **D-E.** Rank-Rank scatter plots illustrating C57 CTR males compared to C57 RCF females vs. C57 CTR females (**D**); DBA CTR females vs. DBA CTR males compared to DBA RCF females vs. DBA CTR females (**E**). All genes in each dataset are ranked based on - log10(padj)*sign(logFoldChange). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Interestingly, in our previous studies, female (but not male) RCF C57 mice were reported to be more sensitive to cocaine effects in adulthood (Di Segni et al., 2017, 2020), while female (but not male) RCF DBA mice were shown to be more vulnerable to depression-like phenotype (Di Segni et al., 2016). Thus, these DEGs may account for the different phenotype observed in adult RCF C57 and DBA female mice. Accordingly, biological processes related to drug addiction (morphine, cocaine, nicotine and amphetamine addiction) were found among the most significantly altered in C57 mice DEGs, suggesting alterations in synaptic neurotransmission. This is corroborated by the significant pathways related to synapses including dopaminergic, glutamatergic and gabaergic synapses, overall representing most of the VTA neurotransmission. Interestingly, some genes (Bdnf, Rgs9, Csf1r, Fgf2, NPY and Drd) involved in mediating synaptic plasticity associated with addiction-like behavioral phenotype (Li and Wolf, 2015; Even-Chen and Barak, 2019; Brady et al., 2019; Anderson et al., 2010) and influenced by early life manipulation (Lo Iacono et al., 2016; Husum and Mathé, 2002) were also found to be altered in adult RCF C57 female mice. In parallel, pathways that are transcriptionally altered by RCF specifically in DBA females may indicate a dysfunction in cellular bioenergetics and metabolic processes (suggested by pathways including oxidative phosphorylation, metabolic pathways and neurodegenerative diseases). A large body of evidence report that mitochondrial dysfunctions in various brain regions are associated with depression and point to a critical role for cellular bioenergetic in the pathophysiology of depression (Bansal and Kuhad 2016; Karabatsiakis and Schönfeldt-Lecuona, 2020). In particular, the mitochondrial complex provides energy to the cells through the ATP production and lower central and peripheral levels (Gardner et al., 2003; Xie et al., 2020) have been strongly associated with depression in both humans and animals (Martins-de-Souza et al., 2012; Wen et al., 2014). To note, genes implicated in mitochondrial electron transport chains, such as NADH-ubiquinone oxidoreductase 1 (ND1), ND2, ATP synthase 5 (ATP5), cytochrome *b-c*1 complex subunit 1 (Uqcrc1) and Uqrcr2 (Gomes et al., 2013) and suggested to be involved in depression (Silva-Costa et al., 2019), were also modulated by our manipulation. The hypothesis of a developmental programming of brain mitochondrial biology (Gyllenhammer et al., 2020) that is controlled by early maternal environment is highly fascinating. Finally, several genes encoding collagen proteins playing an active role in brain architecture establishment, axon guidance and synaptogenesis regulation (Hubert et al., 2009) were altered by RCF manipulation. Consistently, some of them (Col1a2, Cl5a2 and Col27a1) seem to be altered by stress and involved in depression-like phenotype (Smagin et al., 2019).

Our results suggest that, in susceptible genotypes, the long-term effect of an early aversive experience is sexually dimorphic. Future studies will be directed to characterize the nature of these modifications.

Interestingly, we found 75 genes and 4 pathways that are shared among the RCF-induced DEGs in females from C57 and DBA genotypes. We reason that these DEGs could represent "genetically conserved plasticity genes", namely genes that are highly responsive to early environment in females, regardless of genetic background. Not surprisingly, these genes represent in large number processes related to neuronal plasticity and development, such as focal adhesion, axon guidance and extracellular matrix-receptor interaction. Previously studies identified some of the DEGs altered by RCF, such Bdnf (Roth et al., 2009; Boulle et al., 2012; Suri et al., 2013), Gabra (Skilbeck et al., 2018; Mitchell et al., 2018) Fkbp5 (Wang et al., 2018; Criado-Marrero et al., 2019; Ke et al., 2018) and Npy (Yam et al., 2017; Husum and Mathé, 2002; Reichmann and Holzer, 2016) also affected by other early life adversities in different brain areas, including Prefrontal Cortex, Nucleus Accumbens and Hippocampus (Oldham Green et al., 2021; Lo Iacono et al., 2016; Alberry et al., 2020), while other of them, like Pvalb,

are still under investigation (Leussis et al., 2012).

Among the common pathways, it is worth mentioning the PI3K-Akt signalling pathway, a key mechanism mediating the experience-dependent neuronal growth and survival. Notably, decreased Akt activity into the VTA has been proposed to be an important mediator of vulnerability to depression induced by stress exposure (Krishnan and Nestler., 2008).

Sex differences in transcriptional signatures of early and adult stress exposure in many brain regions have been reported in human and animal models (see Brivio et al., 2020 for review; Barko et al., 2019; Labonté et al., 2017; Parel and Peña, 2020 for review). However, to our knowledge, only one pioneering paper investigated the early stress-induced transcriptional alterations in VTA in both sexes of mouse (Peña et al., 2019), and no study reported an almost exclusive effect of early experience in females as, indeed, evident from our results.

Following, we hypothesized that transcriptional changes induced by RCF in female mice could involve sex-specific VTA transcripts, namely genes that are differently expressed between control males and females. Thus, we compared transcriptional profiles of the two sexes in the VTA within each background and obtained two lists of sex-specific DEGs. Of note, these DEGS seem to be highly genotype-dependent, as they overlapped between backgrounds only for few genes (mainly located in sex-chromosomes). However, many of these genes in both backgrounds are described by DAVID analysis as extracellular region constituents, suggesting that different matrix composition and function may underlie the sexual dimorphism and the higher plasticity in female than male VTA.

Comparing sex-specific DEGs with DEGs that were found between RCF and Control females within each background we found a similar percentage (55% for C57 and 57% for DBA) of overlap for both strains. No overlap of these genes was found across the two strains.

We speculate that these DEGs could represent genotype-dependent "female-specific plasticity genes", namely genes biologically primed to respond to environmental manipulation in a sex- and genotype-related manner. Surprisingly the "female-specific plasticity genes" show an 100% inverted directional relationship when the sex-specific DEGs and the RCF-induced DEGs are compared. This is true for both backgrounds, with the exception of the gene Xist in DBA mice. In a simplistic view, sex-specific plasticity genes in females are pushed by RCF toward a "male-like" level of expression. However, this does not correspond to a male-like behavior in adulthood. We speculate that the behavioral dysregulation that we observe in adult females is likely due to the altered "global" gene expression pattern, rather than to the absolute expression level of these genes. According to this view, RCF would act in females preventing the correct female-like gene expression maturation for these groups of genes, which in turn results in a global dysfunction.

Interestingly, in early age, it looks like RCF is making the females behave more like males in the homing test. That is consistent with an interference of RCF with the proper sex-specific transcriptional development.

Future studies will evaluate how early experiences mediate gonadal hormones-dependent transcriptional instructions.

The sexually dimorphic impact of RCF on the VTA could be explained by various independent or complementary scenarios: first, the existence of different developmental paths between males and females (Chocyk et al., 2015; Gillies et al., 2014; Hodes and Epperson, 2019) that may be temporally shifted in their critical plasticity windows for the dopaminergic system development. Female and male brains indeed mature at different rates, and environmental events experienced at the same chronological age could differentially impact neurodevelopmental processes (Bath, 2020).

Second, stress hormones, mainly glucocorticoids, interact with the VTA dopaminergic system to enable the individual to cope with challenging events. In particular, during early life glucocorticoids or stress exposure differentially impact dopaminergic developmental trajectories in male and female brain (Gillies et al., 2014). This differential impact can have long lasting consequences on males and females by setting individual resilience or susceptibility to psychopathology trigged by later stressors throughout the lifespan.

Third, the possibility that sex-specific hormonal "instructions" may make female dopaminergic system more vulnerable to maternally-driven attachment bond-related stimuli. Gonadal steroid hormones represent one of the major drives for sexually dimorphic brain and one mechanism by which early stress can affect DA-related motivated behavior is via regulating gonadal hormones. There is evidence, in fact, for organizational effects of estrogens and androgens on dopaminergic neurons in the VTA and, consequently, on motivated behavior (Eck and Bangasser, 2020; Zachry et al., 2021). Different protocols of early manipulations can increase or decrease estrogen and androgen levels in males and females, thus altering gonadal hormone regulation of the mesolimbic DA system (Eck and Bangasser, 2020). Further works are needed to deeply investigate the hormonal regulatory effects of DA system.

In addition, several studies demonstrate that factors other than sex steroid hormones and glucocorticoids, like different hormonal processes, sex chromosomes themselves and epigenetic events can account for these sex-related differences (Bath, 2020; Gillies et al., 2014; Maccari et al., 2017; Peña et al., 2019; Parel and Peña, 2020).

Finally, an important role for the oxytocin (OXT) in mediating sex-related brain differences would also be taken into account. The neuropeptide OXT plays a role in the offspring development of a bond with a caregiver (Buchheim et al., 2009; Nagasawa et al., 2012; Sharma et al., 2020), and maternal attachment has been linked to development of dopaminergic and OXT neuroendocrine systems (Strathearn et al., 2009; He et al., 2017; Pedersen et al., 1994). Based on this evidence, it is possible to speculate that sex differences in OXT levels within the VTA mediate the sex-related effects of RCF in our study. Further studies, currently in progress in our lab, will investigate this hypothesis.

Investigating epigenetic and molecular pathways regulated by early experiences (Burns et al., 2018) in a sex-specific manner is crucial to understand the mechanisms underlying the clinically described sexual differences (Brivio et al., 2020).

## 5. Conclusions

Here, we report for the first time an extremely higher effect of an early adversity able to interfere with mother-pups attachment bond on females than males from two different genetic backgrounds. We propose to interpret our data within the conceptual models of "differential susceptibility" proposed by Belsky (Belsky et al., 2009). According to Belsky's model, some individuals are more developmentally plastic "for better and for worse"; namely they are more susceptible to environment, regardless of its valence (negative or positive). Within this conceptual framework, our data suggest that females appear to be more "plastic" to environment, thus indicating sex as a critical biological variable in mediating the effects of environmental experiences. Finally, according to "the three-hit concept of vulnerability and resilience" (Daskalakis et al., 2013), we confirm that experiences during early-life can modulate the epigenetic programming of specific brain circuits underlying different aspects of behavioral response to events later in life (Daskalakis et al., 2013) in a genotype dependent manner.

Investigating these genotype dependent- and independent-plasticity genes may help to better understand the molecular basis of clinically reported sex-related psychopathological outcomes as well as the different inter-individual consequences of early adversity exposure in women.

## CRediT authorship contribution statement

**Luisa Lo Iacono:** Conceptualization, Methodology, Investigation. **Camilla Mancini:** Conceptualization, Methodology, Investigation. **Lucy Babicola:** Investigation, Formal analysis. **Marco Pietrosanto:** Investigation, Formal analysis. **Matteo Di Segni:** Conceptualization, Writing –

original draft, Investigation, Formal analysis. **Sebastian Luca D'Addario:** Investigation, Formal analysis. **Diana Municchi:** Investigation, Formal analysis. **Donald Ielpo:** Investigation, Formal analysis. **Tiziana Pascucci:** Methodology, Writing – review & editing. **Simona Cabib:** Methodology, Writing – review & editing. **Fabio Ferlazzo:** Formal analysis. **Francesca R. D'Amato:** Methodology, Writing – review & editing. **Diego Andolina:** Methodology, Writing – review & editing. **Manuela Helmer-Citterich:** Methodology, Writing – review & editing. **Carlo Cifani:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Rossella Ventura:** Conceptualization, Methodology, Writing – original draft, Supervision, Funding acquisition.

## Declaration of competing interest

None.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2021.100406.

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